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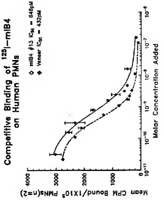
(54) A method for reducing the immunogenicity of antibody variable domains.

(57) A unique method is disclosed for identifying and replacing immunoglobulin surface amino acid residues which converts the antigenicity of a first mammalian species to that of a second mammalian species. The method will simultaneously change immunogenicity and strictly preserve ligand binding properties. The judicious replacement of exterior amino acid residues has no effect on the ligand binding properties but greatly alters immunogenicity.

Fig. 13



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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Solvent exposure of sidechains of framework residues in KOL and J539 Fvs and the residues which occur most frequently at these positions in the various human VH subgroups.

Figure 2. Solvent exposure of sidechains of framework residues in KOL VL and the residues which occur most fraquently at these positions in tha various human V-lambda subgroups.

Figure 3. Solvent exposure of sidechains of framework residues in J539 VL end the residues which occur most frequently at these positions in the various human V-kappe subgroups.

Figura 4. Primers used to isolete DNA encoding murine kappa light chain varieble region and murine IgG2e heevy chain variable region using PCR. Oligodeoxynucleotides used as PCR primers to generate e shortened IgG4 heavy chain. Oligodeoxynucleotides usad in PCR to re-engineer the thymidine kinasa (TK) promotar to facilitate tha expression of the neomycin resistance gene. Oligodeoxynucleotida primers used in PCR to clone the IgH enhancer sequence. Oligodeoxynucleotides used es PCR primers to generete a human keppe light chain constant region.

Figure 5. Oligodeoxynucleotides used in the construction of the "veneered" 1B4 heavy and light chein verieble regions plus those necessary to fuse the humen signal end intronic sequenceds onto these variable regione.

Figure 6. PCR-recombinetion strategy used in the veneering of the 1B4 kappa light chain varieble region.

Figure 7. Outline of the insertion of the "veneered" keppe light chain variabla region and kappe constant region into the light chain expression vector.

Figure 8. PCR-recombinetion strategy used in the veneering of the 1B4 haavy chain variable region.

Figure 9. Outline of the insertion of the "veneered" heevy chein varieble region into the heavy chain expression vector.

Figura 10. Outline of the construction of neomycin selectable expression vector.

Figure 11. Outline of the construction of tha hygromycin selectable expression vector.

Figure 12. Amino acid saquence compleition of the "veneered"-1B4, murine 1B4 and human Gal heavy chain variable regions and the "veneered" 1B4, murine 1B4 and human Len kappa light chain variable ragions. Check marks idicate the individual amino acid residues converted.

Figure 13. Competitive binding assay of native murina 1B4 (opan diamonds) and recombinant "veneered" 1B4 (closed diamonds).

BACKGROUND OF THE INVENTION

The identification and production of murine monoclonal antibodies has lead to numerous tharapeutic applications of these exquisitely spacifc molecules in human diseasa. Tha technologies of molecular biology have further expanded the utility of many antibodies by allowing for the creation of class switched moleculas whose functionality has been improved by the acquisition or loss of complemant fixation. The size of the bioactive molecula may also be reduced so es to increase the tissue target availability of the antibody by either changing tha class from en IgM to an IgG, removing most of the heavy chain constant regIon in the creation of e $F(ab)_2$ or both heavy and light chain constant regions may be dispensed with in the formation of e Fv antibody. Common to all of these potentially therapeutic forms of antibody are the requiste CDRs (complelmentary determining regions) which guide the molecule to its ligand and the framework residues (FRs) which support these letter structures and dictata the disposition of the CDRs relative to one another, Winter European Patent Application, Publication No. 239,400; Riechmann et al., Neture 332: 323-327 (1988). Crystallographic analyses of numeroue antibody structures reveal that the combining site is composed almost entirely of the CDR residues arrenged in e limited number of loop motifs, Padlan and Sheriff, 1990. Tha necessity of the CDRs to form these structures combined with the apprecieted hypervariablity of their primary sequence laads to a greet diversity in the entigen combining site, but ona which hes e finite number of possiblities. Thus, hypermutability end e limited primary sequence repetoire for each CDR would suggest that the CDRs derived for e given antigen from one species of animel would be the same derived from another species. Hance, thay should be poorly immunogenic, if at ell, when presented to e recipient organism in a non- foreign context.

Monoclonal antibody producing hybridomas have been most readily obtained from immunized rodents. Development of similar reagents from human sources has bean frustrated by the currant inability to maintain long tarm cultures of cells which produce sufficient quantities of antibody. Additional problems arise from the regulatory standpoint when cells of human origin are employed for the production of agents to be used in man. These considerations have lead to the widespread use of rodent mono- clonal antibodies for the imaging and treatment of malignancy, prophyllactic administration to guard ageinst toxic shock, modification of graft rejection episodes, and to temper acute inflammatory reactions. In all scenerios where completely rodent or partially rodent (ie,rodent - humen

chimeras) antibodies have been used for therapy the recipients have often illicited an immune response directed toward the antibody. These reactions have limited the duration and effectiveness of the therapy.

Various attempts have been made to minimize or eliminate the immunogenicity of non-human antibodies while perserving their antigen- binding properties. Initially, chimeric antibodies were constructed containing the rodent varibla regions and their associated CDRs fused to human constant domeins. The following references generally describe chimaeric antibody technology: Lobuglio et al., Proc. Natl. Acad. Sci. USA 86: 4220-4224 (1989); United States Patent 4,816,567; PCT International Publication No. WO 87/02671, published May 7,1987; European Petent Publication No. 255,694, published February 10 1988; European Patent Publication No. 274,394, published July 13, 1988; European Patent Publicetion No. 323,806, published July 12, 1989; PCT International Publicetion No. WO/89/00999, published February 9, 1989; European Patant Publication No. 327,000, published August 9, 1989; European Patent Publication No. 328,404, published August 16,1989; and European Patent Publicetion No. 332,424, published September 13, 1989. These proved to be less immunogenic but still approximately half of the recipients mountad en immune responsa to the rodent varieble region framework residues. Furthar reduction of tha "foralgn" nature of the chimeric antibodies has been achieved by grafting only the CDRs from the rodant monoclonal into a human supporting framework prior to ita subsequent fusion with an appropriate constant domain, Wintar European Patant Application, Publication No. 239,400; Riachmann et al., Nature 332: 323-327 (1988). The procedures employed to accomplish CDR-grafting often result in imperfectly "humanized" antibodies. That is to say, the resultant antibody has either lost avidity (usually 2-3 fold, at best) or in an attempt to retain its original avidity e significant number of the murine framework residues have replaced the corresponding ones of the chosen human fromework. In this later case, the immunogenicity of the modified "humanized" antibody is difficult to anticipate a priori.

The ligand binding characteristics of an antibody combining site are determined primarily by the structure and relative disposition of the CDRs, although some neighboring residues also have been found to be involved in entigen binding (Davies et al., Ann. Rev. Biochem. 59: 439-473 [1990]). Fine specificity can be perserved in e "humanized" antibody only if the CDR structures, their interaction with each other, and their interaction with the rest of the variable domains are strictly maintained. One may anticipate that the key residues represent "interior" and interdomain contact residues, hence those surface exposed residues which are immediately available for immune surveillance should be non- inclusive of the structural residues.

OBJECTS OF THE INVENTION

It is, accordingly, an objective of the present invention to provide a means of converting a monoclonal antibody of one mammalian species to a monoclonal antibody of another mammalian species. Another object is to identify the amino acid residues responsible for species specificity or immunogenicity on the exterior of the monoclonal antibody. Another object is judiciously raplace or veneer the exterior amino acid residues of one species with those of a second speclas so thet the antibodies of the first species will not be immunogenic in the second spacies. A further object is to make replacements only in fremework regions of the heavy and light chains of the antibody molecule and not in the complementarity-determining regions. Another object of the invention is to provide novel DNA sequences incorporating the replacement amino acid residues. Another objact is to provida a vector containing the DNA sequences for the altered antibody. Another object is to provide a eukaryotic or procaryotic host transformsd with e vector containing the DNA sequenca for the veneered antibody.

SUMMARY OF THE INVENTION

A unique method is disclosed for identifying and replacing immunoglobulin surface amino acid residues which converts tha antigenicity of a first mammalian species to that of a second mammelian species. The method will simultaneously chenge immunogenicity and strictly preserve ligind binding properties. Tha judicious replacement of exterior amino acid residues has no effect on the ligind binding properties but greatly elters immunogenicity.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a "humanization" procedure which simultaneously reduces the immunogenicity of the rodent monoclonal antibody while perserving its ligand binding properties in their entirety. Since the antigenicity of a protein is primarily dependent on the neture of its surface, the immunogenicity of an xenogenic or allogenic antibody could ba reduced by replacing the exposed residues which differ from those usually found in another mammalian species antibodies. This judicious replacement of exterior resi-

dues should have little, or no, effect on the interior domains, or on the interdomain contacts. Thus, ligand binding properties should be unaffected as a consequence ot alterations which are limited to the variable region tramework residues. The process is retered to es "veneering" since only the outer surface or skin ot the entibody is eltered, the supporting residues remain undisturbed.

The procedure for "veneering" makes use of the available sequence data tor human antibody variable domains complied by Kabet et al., "Sequences of Proteins of Immunological Interest", 4th ed., Bethesda, Maryland: National Institutes of Health, 1987, updates to this database, and other accessible U.S. and foreign databases (both nucleic acid and protein). The subgroups Into which the various sequences have been combined ere presented in Figures 1 - 3, indicating the most frequently occurring amino ecld et each framework position. Also presented are the sequences of the verious J-minigenes. The solvent accessibilities of the emino acids, es deduced from the known three-dimensionel structure for human end mouse antibody fregments, ere Included in these figures.

High resolution X-rey crystallography of the verieble domaine of the antibodies KOL and J539 heve been subjected to extensive refinement beginning with the structures evelleble from the Protein Data Bank (Bernstein et el., J. Mol. Biol. 112: 535-542 1977; file 2FB4 for KOL end file 2FBJ for J529). The solvent eccessibilities were computed es described by Pedlan Proteins: Struct. Funct. Cenet. 7: (1990).

There ere two steps in the process of "veneering". First, the fremework of a first animel species, I.e. the mouse, variable domains are compared with those corresponding frameworks of a second animel species, I.e. human. It is intended thet this invention will ellow the entigenic elteration of any animel species entibody. The present invention is illustrates the conversion of murine antibody to human antibody, but this is for illustrative purposes only. The most homologous human variable regions are then compered residue for residue to the corresponding murine regions. This will also define the human subgroup to which each mouse sequence most closely resembles. Second, those residues in the mouse tramework which differ from its human counterpart are replaced by the residues present in the human counterpart. This switching occurrs only with those residues which are at least partially exposed (mE and Ex; Figures 1-3). One retains in the "veneered" mouse antibody: its CDRs, the residues neighboring the CDRs, those residues defined as buried or mostly buried (mB and Bu; Figures 1-3), and those residues believed to be involved with interdomain contacts (boldface, Figures 1-3).

Human and murine sequences frequently differ at the N-terminus of both heavy and light chains. The N-termini ere contiguous with the CDR surface and are in position to be involved in ligand binding. Thus, wisdom would dictate that these murine termini be retained in its "veneered" version.

Finally, repalcement of some amino acid types could have a significant effect on the tertiary structure or electrostatic interactions of the variable region domains. Hence, care should be exercised in the replacement of proline, glycine, and charged amno acids.

These criteria and the following procedures are used to prepare recombinant DNA sequences which incorporate the CDRs of a first mammalian species, animal, mMAb, both light and heavy chains, into e second mammalian species, human, eppearing frame works that can be used to trensfect mammalian cells for the expression of recombinant human antibody with the entigen specificity of the animal monoclonal antibody. The present invention further comprises a method for constructing and expressing the eltered entibody comprising: (i) mutagenesis and assembly of verieble region domains including CDRs end mutagenesis and assembly of variable region domelns including CDRs end FRs regions; (ii) preparetion of an expression vector including et least one verieble region which upon transfection into cells results in the secretion of protein sufficient for avidity end specificity determinetions; and (iii) co-amplificetion of heevy end light chain expression vectors in appropriate cell lines. The present invention provides recombinent methods for incorporeting CDRs from animal monoclonal entibodies into frameworks which eppear to be human immunoglobulin in nature so that the resulting recombinant entibody will be either weakly immunogenic or non-immunogenic when edministered to humans. Preferably the recombinant Immunoglobulins will be recognized as self proteins when administered for threapeutic purposes. This method ot "veneering" will render the recombinant entibodies useful as therapeutic agents because they will be either weakly immunogenic or non-immunogenic when administered to humans. The invention is further contemplated to include the recombinant conversion of any animel monoclonel antibody into a recombinant "human-eppearing" monoclonal antibody providing that a suitable framework region can be identified (as described below). The enimal monoclonals mey include, but ere not limited to, those murine monoclonal antibodies described by Van Voorhis et al., J. Exp. Med. 158: 126-145 (1983) which bind to human leukocytes and the eppropriate mMAbs produced by hybridomas deposited in the Hybridoma Cell Bank maintained by the American Type Culture Collection (ATCC) and

described in the ATCC Catalog of Cell Lines 8 Hybridomas, No. 6, 1988.

The CDR sequences from the animal monoclonal antibody are derived as follows. Total RNA is extracted from the murine hybridomas, for exampe the 1B4 myeloma cells described by Wright et al., Proc. Natl. Aced. Sci. USA 80: 5699-5703 (1983), the 60.3 cells described by Beatty et al., J. Immunol. 131:2913-2918 (1983), the TS1/18 cells described by Sanchez-Medrid et al., J. Exp. Med. 158: 1785-1803 (1983), and other anti-CD18 or CD11 monoclonal antibodies and hybridomas as described in Leukocyte Typing III, Springer-Verlag, New York (1988), using standard methods involving cellular solubilization with guanidinium isothiocyanete (Chirgwin et al., Biochem. 18: 5294-5299 [1979]).The murine 1B4 mMAb will be used es tha primary example of enimal MAb that can be "veneered" by the unique process being disclosed. The Invention is intended to include the conversion of any animal immunoglobulin to e "human-appearing" Immunoglobulin. It ia further intended that "human-appearing" Immunoglobulin (Ig) can contain either kappa or lambde light chains or be one of any of the following heavy chain isotypes (alpha, delta, epslon, gemma and mu).

Pairs of degenerate oligodeoxynucleotide primers (Figure 4) representing sequences within framework 1 of the murine kappa light chain variable region and light chain constant domain, or those within framework 1 of the murine IgG2a heavy chain varieble region and heavy chein constant CH1 domein are synthesized on an Applied Biosystem 381A DNA synthesizer, removed from the resin by treatment with concentrated NH_4OH and desalted on a NAP-5 column eluted with H_2O. Total RNA, about 2 µg, Is reverse transcribed for 30 min at 42°C uaing Moloney MLV reverse transcriptase, about 200 units (BRL), and about 10 pmoles of the constant region complementary strand primers for either the heavy or light chain. The reverse transcriptase Is heat Inactivated, about 95° C for about 5 min, and the reactions are made to contain in ebout 100 µl of PCR buffer about 50 pmoles of each of the paired primers and and 2.5 units of Teq polymerase. About 45 cycles of amplificetion (2', 94° C; 2', 55° C; 2' 72° C) are followed by gel purification of the enticipated 400+ base pair (bp) DNA fragments. Prior to subcloning those DNAs into a blunt-ended intermediate plasmid such as pSP72 (Promega) they are terminally phosphorylated using T4 polynucleotide kinase. Multiple clones representing these PCR emplified sequences are grown and submitted to DNA sequence determinations using Sequenase® and T7 end SP6 specific sequencing primers. A unique DNA sequence representing a murine IgG2a heavy chain variable region is obtained by analysis of the derived amino acid sequences. Replacement of the "murine-appearing" framework residues with those residues compatible with a human variable region is accomplished utilizing the following unique processes. An appropriate human framework is determined utilizing the criteria discussed below. The light chain variable region framework with sufficient homology to the the m1B4 framework was determined to be the human LEN framework (FR). The Len FR shows a similarity of 90% and an identity of 81% when compared to murine 1B4. This sequence, with its leader, 3' intronic sequences and engrafted m1B4 CDRs had been subcloned into the intermediate vector pGEM3Z (Promega), as described in Daugherty et al. Nucleic Acids Res. 19: (1991). About eight oligodeoxynucleotide primers (Figure 5) are synthesized representing the primers necessary to generate by polymerese chain reaction (PCR) emplification four DNA fragments. Incorporeted into all but the terminal oligodeoxynucleotide primera were those sequences corresponding to the veneered MAb 1B4 light chain, with its unaltered CDRs, and at least 15 bases of 5'-terminal complementarity to allow for the subsequent PCR- directed recombination of these four fragments. For the purposes of exemplifying the "veneering" process the LEN light chain variable region already containing an engrafted set of CDRs representing those within the light chain of murine 1B4 was used as the template into which mutations were pleced so aa to easily create the "veneered" framework sequence. The appropriate primer pair (S1 & V9, V10 & V11, etc.), about 50 pmole each, was combined with about 10 ng of plasmid DNA representing the LEN CDR-grafted framework, ebout 2.5 units of Taq DNA polymerase and about twenty-five (25) cycles of PCR amplification ensued (cycle periods: 1', 94° C; 1', 55° C; 2' 72° C). The products of the four reactions, purified by agarose gel electrophoresis, are combined, about 10 ng of each DNA fragment, along with terminal oligodeoxynucleotide primers (A1 &A2, Figure 6) and Teq DNA polymerase. The combined fragments were PCR amplified (25 cycles of: 2', 94° C; 2', 55° C; 2' 72° C). Following restriction endonuclease digestion with Hind III and Xba I the amplified DNA is purified by agarose gel electrophoresis and subcloned into compatible sites of an intermediate vector pSP72 (Promega) which contains the human kappa light chain constant region (see Figure 7). Genomic DNA, about 1 µg, purified from a human B cell line (GM0108A: NIGMS Human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, NJ) is used as a template for PCR amplification (Figure 4) of about a 920 base pair fragment containing the splice acceptor for the kappa light chain constant domain, the exon and e portion of its 3'-untran-

slated region. The PCR product is purified by agarose gel electrophoresis, digested with Bam H1 endonuclease, and subcloned into pSP72 previously linearized with Bam H1. The individual clones representing the pSP72 intermediate vector containing both the 1B4 "veneered" light chain variable region and the human kappa constant region derived by PCR amplification of human DNA are used to determine the DNA sequence of the "veneered" light chain varisble region.

The "veneered" heavy chain portion of the recombinant antibody is derived from the mutated version of the marine 1B4 heavy chain variable region fused to the human constant region of a gamme 4 subtype obtained from a lambda library constructed by Flanegen end Rabbits, Nature 300: 709-713 (1982). The verlable region of the "veneered" heavy chain is constructed from five DNA fragments representing a signal sequence, portions of the mutated murine heavy chain variable region, and an intronic sequence (Figure 8). Oligodeoxynucleotide primer pairs (Figure 5) are synthesized representing the primers necessary to generate by PCR amplification these five DNA fragments from about 10 ng of plasmid DNA templete obtained from a pSP72 intermediate vector containing the heavy chein varieble region previously used to determine the murine 1B4 CDR sequence. Amplification of the signel fragment, variable region fragments, and intron-conteining fragment was as described above. The agerose gel purified products are combined, ebout 10 ng of eech product, with terminal oligodeoxynucleotide primer pairs (Figure B) and the PCR-genereted in vitro recombined template is amplified using the standard procedures described above. Prior to subcloning into a Hind III and Bam HI digested expression vector containing the human heavy chain gamma 4 constant region (Figure 9), this recombined product is similarly digested and agarose gel purified. Individual clones ere submitted to DNA sequence determination using Sequenase® and T7 and SP6 specific sequencing primers end one is chosen for subsequent expression. The gemma 4 heavy chain constant region is subcloned as about a 6.7 Kb Hind 111 fragment derived from the plasmid pAT84 into the Kind III site of the intermediate vector pSP72. This plasmid is then used as the template DNA from which a shortened version of the gamma 4 constant region is subcloned using PCR amplification and the primer pairs indicated in Figure 4. Eukaryotic expression vectors are constructed as described below.

Expression vectors are defined herein as DNA sequences that are required for the transcription of cloned copies of genes and the translation of their mRNAs in an appropriete host. Such vectors can be used to express eukaryotic genes in a variety of hosts such as bacteria, blue-green algae, plant cells, yeast cells, insect cells and animal cells. The immunoglobulins may also be expressed in a number of virus systems. Specifically designed vectors allow the shuttling of DNA between host such as bacteria-yeast or bacteria-animal cells. An appropriately constructed expression vector should contain: an origin of replication for autonomous replication in host cells, selectable markers, a limited number of useful restriction enzyme sites, a potential for high copy number, and strong promoters. A promoter is defined as a DNA sequence that directs RNA polymerase to bind to DNA and initiate RNA synthesis. A strong promoter is one which causes mRNAs to be initiated at high frequency. Expression vectors may include, but are not limited to, cloning vectors, modified cloning vectors, specifically designed plasmids or viruses. The heavy chain immunoglobulin molecule is transcribed from a plasmid carrying the neomycin (G418) resistance marker while the light chain immunoglobulin is transcribed from a plasmid carrying the hygromycin B resistance marker. With the exception of the drug resistance portion of these plasmids they are identical. The preferred progenitor of the immunoglobulin expression vectors is the pD5 (Berkner and Sharp, Nucl. Acids Res. 13: 841-857 [1985]) eukaryotic expression vector which contains the origin of edenovirue replication, the SV40 enhencer domain, the edenovirus mejor late promoter, the adenovirus 2 tripertite leader, a 5' splice donor from the edenovirus third leader end a 3' splice acceptor derived from an immunoglobulin locus, a multiple cloning site placed in the Bam H1 site subsequent to receipt of the vector, and the SV40 late polyadenylation signal (Figure 10). The origin of replication is removed by digestion with Eco R1 and Kpn I and replaced by two fragments representing the neo selectable marker gene (derived from plasmid pCMVIE-AK1-DHFR as en Eco R1/Bam H1 about 1.B Kb fregment) and the Ig heavy chain enhancer (obtained as a PCR amplified fragment using human DNA es the template, and the oligodeoxynucleotides listed in Figure 4 as the primer pair, following its digestion with Bgl II and Kpn 1). The resultant expression vector is found to lack e small portion of the TK promoter responsible for the transcription of the neomycin gene. This is replaced by insertion into the Eco RI site about a 0.14 Kb PCR amplified fragment derived from the CMVIE-AK1-DHFR DNA using the primer pair listed in Figure 4. The resultant heavy chain expression vector (p8941) is modified by removal of the indicated Hind III and Xba I sites using standard procedures. To convert this vector into one expressing the hyg romycin B selectable marker the neomycin-resistance cassette is removed by digestion first with Eco R1 followed by

DNA polymerase-directed fill in of the 5' overhand, then subsequent Sal I digestion. The about 1.9 Kb hygromycin B expression cassette, TK promoter and TK polyadenylation signal flanking the hygromycin B gene, (obtained as a 1.8 kb Bam H1 fragment in plasmid pL690, Gritz and Davies, Gene 25: 179-188 [1981]) is removed from the plasmid pAL-2 by Bam H1 digestion and subcloned into the Bam H1 site of the intermediate vector pSP72. The hygromycin B cassette is removed from this vector by digestion with Sma I and Sal I and cloned into the expression vector linearized as described above to create a blunt end and Sal I end DNA fragment (Figure 11).

Expression of the 1B4 "veneered" kappa light chain is accomplished by transferring this cistron from the pSP72-based intermediate cloning vector (p8952), containing the human kappa constant region, to the hygromycin B selectable eukaryotic expression vector (Figure 7). An about 1.5 kb DNA fragment resulting from the endonuclease digestion of p8952 with Spe I and Cla I is purified by agarose gel electrophoresis and ligated into the expression vector which has previously been linearized, following digestion with the same two restriction enzymes, and agarose gel purified. The heavy chain eukaryotic expression vector is constructed in two steps. First, the p8950 vector containing the modified heavy chain variable region of murine 1B4 fragment is digested with Bgl II and Bam H1. The agarose gel purified 0.75 kb fragment is ligated into the Bam H1 site of the p8941 vector and recombinant clones containing this fragment in the proper orientation are identified. Plasmid DNA from one such clone is linearized by Bam H1 digestion and ligated with a 1.78 Kb Bam H1 fragment representing a short version of the human gamma 4 constant region, derived from plasmid pAT84 by PCR amplification. Following the identification of clones containing these inserts in the appropriate orientation, plasmid DNAs (one which is referred to as p8953) are grown and purified for transfection into recipient mammalian cells. Equal amounts, about 10 µg, of the plasmids encoding the 1B4 "veneered" IgG4 heavy chain and the 1B4 "veneered" kappa light chain are transfected by standard calcium phosphate precipitation procedures into the monkey kidney cell line CV-1P or the human embryonic kidney cell line 293. The culture supernants, assayed by a trapping ELISA (described below), were found to contain a human kappa light chain / human IgG4 immuno-globulin. Immulon-2 (Dynatech Labs.) 96-well plates are coated overnight with about a 5 µg/ml solution of mouse anti-human kappa chain constant domain monoclonal antibody (cat. #MC009, The Binding Site, Inc., San Diego, CA) in about 0.1 M $NaHCO_3$ buffer (pH 8.2) at about 4° C, and blocked with about 1% bovine serum (BSA) in about 0.1 M $NaHCO_3$ for about 1 hour at about 25° C. After this and all subsequent steps, washing was performed with phosphate buffered saline (PBS). The wells are then challenged with conditioned medium containing recombinant anti-CD18 antibody, or with predetermined quantities of human IgG4/keppa purified by protein A Sepharose (Pharmacia Fine Chemicals) chromatography from human IgG4 myeloma serum (cat. # BP026, The Binding Site, Inc.) All samples are diluted in PBS containing about 0.05% Tween-20. About 100 µl aliquots are incubated for about 1 hour at about 37° C in triplicate, and standard calibration curves are constructed using IgG4 concentrations ranging from about 10 ng/ml to about 100 ng/ml. Bound and fully assembled human IgG4 (either native or the recombinant 1B4 human "veneered" IgG4 constructs) are detected with about 100 µl aliquots of a 1:500 dilution of mouse anti-human IgG4 Fc monoclonal antibody conjugated to alkaline phosphatase (cat #05-3822, Zymed Laboratories, Inc.) in phosphate buffered saline (PBS) containing about 1 % BSA. After incubation for about 1 hour at about 37° C and subsequent washing, the quantities of bound conjugate are detected by incubating all samples with a 1 mg/ml solution of p-nitrophenyl phosphate in 0.1 M 2,2' amino methylpropanediol buffer, pH 10.3, for about 30 minutes at about 25° C. The adsorbance of the wells is determined with a UV Max ELISA plate reader (Molecular Devices) set at 405 nm. The antibody secreted by the transfected human 293 cells or monkey kidney CV1 P cells, either following transient expression or subsequent to stable clone isolation, is isolated by protein A chromatography, the concentration of recombinant human anti-CD18 antibodies determined by the trapping Elisa described above, and used to compete with the binding of radiolabeled murine 1B4 to the CD18 ligand on the surface of activated human PMNs. Affinities of r-anti-CD18 antibody constructs are determined using a competitive ^{125}I-1 B4 soluble binding assay with stimulated human polymorpho-nuclear leukocytes (PMNs). Purified murine anti-CD18 monoclonal antibody (50 ug) is iodinated using chloramine-T (Hunter, W.M. and Greenwood, F.C., Nature 194: 495-496, 1962), and the radiolabeled antibody purified using a Bio-Sil TSK250 (Biorad, Richmond, CA) gel filtration HPLC column (which fractionates proteins in the range of 1-300 x 103 daltons) equilibrated in 0.1 M phosphate buffer, pH 7.0. Effluent radioactivity is monitored with an in-line detector (Beckman Model 170; Beckman, Fullerton,CA) and total protein measured at OD_{280} with a Kratos Spectroflow 757 detector (Kratos, Mawah, N.J.). A single ^{125}I-1B4 peak composed of coincident OD_{280} and radioactivity tracings char-

acteristically elutes at about 6 minutes, 30 seconds following sample injection. Specific activity of the product is generally about 10 µCi/µg protein, and 97-99% of the counts are precipitable with 10% trichloroacetic acid. The binding of this radiolabeled antibody is assessed on human PMNs purified on a discontinuous Ficoll/Hypaque gradient (English, D. and Anderson, B.R., J. Immunol. Methods 5: 249-255, 1974) and activated with about 100 ng/ml phorbol myristate acetate for about 20 minutes at about 37°C (Lo et al., J.Exp. Med. 169: 1779-1793, 1989). To determine the avidity of antibodies for CD18 molecules on the PMN surface, about 1 x 10^5 activated PMNs are incubated in a buffer such as Hanks balanced salt solution containing about 20 mM Hepes (pH 7.2), about 0.14 units aprotinin (Sigma Chemical Co.) and about 2% human serum albumin (binding buffer) containing about 1.3 ng ^{125}I-1B4 (2.8×10^{-11} M) in the presence of increasing concentrations of unlabeled 1B4 antibody (about 10^{-7} to 10^{-15}M) in about a 300 µl reaction volume for about 1 hour at about 4°C with constant agitation. Cell bound 1B4 is separated from the unbound antibody by centrifugation through a 0.5 M sucorse cushion (4,800 x g, 3 minutes); the tubes are frozen on dry ice, and the tips cut off and counted with an LKB gamma counter. The IC_{50} of the anti-CD18 antibody for the inhibition of ^{125}I-1B4 antibody binding is calculated using a four parameter fitter program (Rodbard et al., In, "Radioimmunoassey and Related Procedures in Medicine", International Atomic Energy Agency, Vianna, vol 1,469 - 504, 1978). The affinity of the "veneered" r-anti-CD18 antibody for the CD18 ligand is determined in a similar manner using murine ^{125}I-1B4 antibody and increasing quantities, as determined by the trapping Elisa, of unlabeled r-anti-CD18. The results of the binding assays are shown in Figure 13 and indicate that the avidity of the "veneered" recombinant 1B4 antibody is equal to that of the murine 1B4 monoclonal antibody. This result shows that an antibody with presumptive human isotype may be recombinantly constructed from the murine parent antibody by the introduction of numerous point mutations in its framework residues and expressed fused to human kappa and gamma 4 constant domains without loss in avidity for the antigen. It can be inferred from this result that the point mutations within the framework regions do not alter the presentation of the murine 1B4 light chain and heavy chain CDRs. Many of the examples of construction of recombinant human antibodies containing complementarity regions replaced by those found within murine monoclonal antibodies have resulted in loss of avidity for the ligand or antigen. Thus, although these latter transmutations are possible, the successful maintenance of avidity is not assured. This procedure described above demonstrates that when strict attention is payed to the framework regions, and the nature of the amino acids within each framework, "humanization" may potentially be achieved without the loss of avidity which accompanies the transfer of CDRs to the "generic" human frameworks ("humanization") employed by Winter, European Patent Publication No. 239,400, published September 30, 1987.

To identify human framework sequences compatible with the CDRs of, say, murine 1B4, human frameworks with a high degree of sequence similarity to those of murine 1B4 are identified. Sequence similarity is measured using identical residues as well as evolutionarily conservative amino acid substitutions. Similarity searches are performed using the murine 1B4 framework sequence from which the CDR sequences had been removed. This sequence is used to query a database of human immunoglobulin sequences that had been derived from multiple sources. Sequences with a high degree of sequence similarity are examined individually for their potential as humanizing framework sequences. In this way, the human homologue providing the murine CDRs with the structure most similar to their native murine framework is selected as the template for the construction of the "veneered" variable regions (Figure 12). Should human frameworks of sufficient similarity not be identifiable from compiled sequences, it is possible to isolate from human genomic DNA a group of closely related variable regions using recombinant technology. Thus, a degenerate 5' upstream oligodeoxynucleotide primer may be designed from the conserved sequences within the amino-terminus of each of the various human FR1 regions and paired with a degenerate 3' downstream oligodeoxynucleotide primers fashioned from the FR sequence determined from the murine monoclonal whose CDRs one wishes to transfer into a human context. These primer pairs are then used to PCR amplify from a human genomic template those DNA sequences which are flanked by the primer pair. The resulting DNAs may then be cloned and the DNA sequence derived from individual members will describe various murine-related human variable regions. The paucity of somatic mutations in framework residues and the conservation of amino acid sequence between mouse and man make this approach possible.

The construction of a complete recombinant human IgG4 antibody, whose heavy and light chain variable domains contain the CDR residues of the murine monoclonal antibody, with complete retention of the specificity and avidity of the parent murine monoclonal antibody is disclosed. The construction of the "veneered" light chain framework derived from the human sequence of LEN fused

with a human kappa light chain constant region is described above. The murine variable region framework sequence, devoid of CDR sequences, is used to query a database of complete human variable region sequences. The human sequences that are most similar to the murine framework region are then analyzed individually to determine both their sequence identity and similarity to the murine framework region. In the case of murine 1B4 these sequences include, but are not limited to, "Gal", chosen because of its high degree of both similarity and identity with the 1B4 heavy chain sequence. The Gal FR has been found to be 85% similar and 79% identical to murine 1B4. These values are based upon the Dayhoff similarity matrix of evolutionarily conserved amino acid substitutions (R. M. Schwartz, M. O. Dayhoff, in Atlas of Protein sequence and structure M. O. Dayhoff, Eds. (National Biomedical Research Foundation, Washington, DC [1979]) (Figure 12). To prepare a recombinant DNA encoding the murine heavy chain CDRs in the context of a human-appearing framework the following procedures are performed. A set of ten short oligodeoxynucleotides are synthesized. Each pair is combined in a separate PCR reaction with the DNA template representing the murine 1B4 heavy chain variable region, amplified and isolated following PCR of the RNA of the murine hybridome 1B4 as described above. Thus, about 50 pmole of each primer pair was combined with about 10 ng of plasmid DNA representing the murine 1B4 heavy chain variable region, about 2.5 units of Taq DNA polymerase and about twenty-five (25) cycles of PCR amplification ensued (cycle periods: 1', 94°C; 1', 55°C; 2' 72°C). The products of the five reactions (Figure 8) encoded portions of the 1B4 heavy chain variable region, beginning with the signal peptide encoding region and ending with the 3' intronic sequence which resides between the variable region coding domain and the IgG4 constant region sequence, with the desired point mutations to create a "veneered" variable region framework. These five fragments are purified by agarose gel electrophoresis, combined, about 10 ng of each DNA fragment, along with terminal oligodeoxynucleotide primers (A1 &A2, Figure 5) and Taq DNA polymerase. The combined fragments were PCR amplified (25 cycles of: 2', 94°C; 2', 55°C; 2' 72°C). By virtue of the complementary ends of the five fragments, the polymerization/denaturation/polymerization cycles of the polymerase chain reaction result in the formation, and subsequent amplification, of the combined sequences. Following 25 cycles of amplification the combined 0.8 Kb fragment is electrophoretically purified from an agarose gel and was digested with restriction enzymes Spe I and Bam H1. Following agarose gel electrophoresis, the purified DNA fragment is ligated into the heavy chain expression vector, p8958 (see Figure 9), in place of the chimaeric variable region existing in this vector. Each "veneered" variable region, with its associated human constant region, residing within a pD5-based expression vector plasmid was co-transfected into 293 cells and CV1 P cells and recombinant human antibody is found to be present in the conditioned medium 48 hours post transfection. The "veneered" recombinant antibody is isolated by protein A chromatography. The avidity of this antibody for the CD18 ligand displayed on the surface of activated human PMNs is compared with that of the murine 1B4 monoclonal antibody parent. Figure 13 shows that although each antibody contains the same set of six CDRs within different framework domains, they exhibitidentical avidity for the ligand. Thus, the avidity of an antibody molecule does not rely upon the variable region framework residues which are surface exposed, rather the proper structure in which the CDRs are presented must be significantly influenced by the buried and inter/intra active residues. The parent murine monoclonal antibody demonstrates an IC_{50} of about 1.0 to about 0.7 nM, the "veneered" molecule has a similar IC_{50}.

This invention further relates to a method of inhibiting the influx or migration of leukocytes capable of expressing CD18 antigen (leukocyte integrin, beta subunit) on their surface into a site of inflammation or a tissue area or organ that will become inflamed following an influx of the cells. The inflammation which is the target of the method of the present invention may result from an infection with pathogenic microorganisms such as gram-positive and gram-negative bacteria, parasites and fungi. The response may also be induced by viruses and non-infectious means such as trauma or reprefusion following myocardial infarction or stroke, immune responses to foreign antigen and autoimmune responses. The recombinant human anti-CD18 antibodies are useful in the treatment of inflammation in lung, central nervous system, kidney, joints, endocardium, eyes, ears, skin, gastrointestinal tract and urogenital system. Disease states in which the recombinant human anti-CD18 antibodies are useful as therapeutic agents include, but are not limited to: infectious diseases where active infection exists at anybody site, such as meningitis; conditions such as chronic or acute secondary inflammations caused by antigen deposition; and other conditions such as, encephalitis; arthritis; uveitis; colitis; glomerulonephritis; dermatitis; psoriasis; and respiratory distress syndrome associated with sepsis and/or trama. Other inflammatory diseases which may be responsive to recombinant human anti-CD18 antibody include, but are not limited to, immune disorders and conditions involv-

ing T-cell and/or macrophage attachment/recognition, such as acute and delayed hypersensitivity, graft vs. host disease; primary autoimmune conditions such as pernicious enemia; infection related autoimmune conditions such as Type I diabetes mellitis; fleres during rheumatoid arthritis; diseases thet involve leukocyte diapedesis, such as multiple sclerosis; antigen-antibody complex mediated diseases including certain of the secondary infection states listed above; immunosuppression; and transplent rejection. Inflammatory conditions due to toxic shock or traume such as adult respiratory distress syndrome and reperfusion injury; and disease states due to leukocyte dyscrasias and metastasis, are included within the scope of this invention. The present invention is also applicable to the inhibition of leukocyteendotheliel attachment for diagnostic and therapeutic purposes; such as the iatrogenic opening of the endothelium to prevent the ingress of leukocytes during the ingress of a therapeutic drug in the instance of chemotherapy; or to enhance the harvesting of leukocytes from patients.

Recombinant human anti-CD18 antibodies or an active fragment thereof can be used to treat the above mentioned diseeses. An active fragment will include the F(ab')2, the Feb and any other fragment that can bind to the CD18 entigen. Recombinant humen anti-CD18 antibodies can be administered alone for non-infectious disease states or combined with antibiotics or other anti-infective agents for the treatment of infectious diseases for reasons discussed above. Administration will generally include the antibodies and possibly other substances in e physiologically acceptable medium or pharmaceutical cerrier. Such physiologically acceptable media or phamaceutical carriers include, but are not limited to, physiologicel saline, phosphate buffered saline, phosphate buffered saline glucose, buffered saline and the like. The antibodies and any anti-infective agent will be administered by parenterel routes which include intravenous, intramusculer, subcutaneous and intraperitoneal injection or delivery. The amount of the antibodies and the mixture in the dosage form is dependent upon the particular disease state being treated. The amount of the recombinant human anti-CD18 antibody utilized in a dosage form can range from about 1 to about 1,000 mg, with a range of from about 10 mg to about 100 mg being preferred. The antibodies can be administered daily or less than daily as determined by the treating physician. The following examples illustrate the present invention without, however, limiting the same thereto.

EXAMPLE

Preparation of a "Veneered" Recombinant Antibody

An antibody was produced in which the variable domain of the light chain comprises the framework region of a murine light chain modified to contain surface exposed amino acids of human derivation. The varieble domain of the heavy chain is similarly derived from the murine heavy chain with point mutations which replace murine exposed residues with human-appearing residues. The light chain human framework region was derived from human myeloma protein LEN. The CDR and framework sequences from the murine monoclonal antibody 1B4 which binds to CD18 (the beta subunit of the leukocyte integrin B-2 family which includes: LFA-1, Mac-1, and p150.95) were derived as follows. The hybridoma designated 1B4 which produces 1B4 monoclonel entibody wes deposited under the Budapest Treaty at the International Depository Authority: American Type Culture Collection, 12301 Perklawn Drive, Rockville, MD, 20852. Viability was determined on June 8,1989 and the hybridoma was designeted HB 10164. Previous experiments had determined this antibody to be en IgG 2a with e kappa light chain (Wright et el., Proc. Natl. Acal. Sci. USA 80: 5699-5703 [1983]).

Total RNA was extracted from the 1B4 myeloma cells using standard methods involving cellular solubilization with guenidinium isothiocyanate (Chirgwin et al., Biochem. 18:5294-5299 [1979]). Sets of degenerate oligodeoxynucleotide primers (Figure 4) representing sequences within framework 1 of the murine kappa light chain variable region and kappa light chain constant domain, or those within framework 1 of the murine IgG2a heavy chain verieble region and heavy chain constant CH1 domain were synthesized by standard phosphoramidite procedures on an Applied Biosystem 381A DNA synthesizer. Removal of the oligodeoxy-nucleotides (oligos) from the resin was accomplished by treatment with concentrated NH_4OH followed by desalting on a NAP-5 column (Pharmacia) with H_2O elution (when the oligos were <45 bases in length), or by use of an OPC column (Applied Biosystems Inc) with 20% acetonitrile elution (when the oligos were >45 bases in length), as recommended by the manufacturers. Total RNA (2μg) was reversed transcribed for 30' at 42°C using Moloney MLV reverse transcriptase (200 units, BRL) and 10 pmoles of the constant region complementary strand primers representing either heavy or light chein in e buffer (finel volume of 20 μl) containing 50 mM Tris HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, and 20 units of RNAsin (Pharmacia). The reverse transcriptase was heat inactivated (95°C, 5') and the reactions were made to contain in 100μl of

PCR buffer (10 mM Tris HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM each dNTP), 50 pmoles of each of the paired primers, and 2.5 units of Taq polymerase (Perkin Elmer/Cetus). Polymerase chain reaction (PCR) amplification was carried out essentially as described by Saiki et al., Science 230: 1350-1354 (1985) and others (Mullis et al., Cold Srping Harbor Symp. Quant. Biol.51: 263-273 [1986], Dawasaki and Wang, PCR Technology, Princples and Applications for DNA Amplification, Erlich, Ed., Stockton Press, NY, pp. 89-97 [1989], Tung et al., ibid. pp. 99-104 [1989]). Forty five cycles of amplification by a DNA Thermal Cycler (Perkin Elmer Cetus Instruments)(2', 94°C; 2', 55°C; 2' 72°C) were followed by gel purification of the anticipated 400+ base pair (bp) DNA fragments. Prior to subcloning the DNAs into a blunt-ended intermediate plasmid (pSP72, Promega) they were terminally phosphorylated using T4 polynucleotide kinase (Boehringer Mannheim). Multiple clones representing these PCR amplified sequences were isolated form DH5 transformed E.coli plated on LB agar plates containing 50 μg/ml ampicillin, grown by described procedures (Maniatis et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982), plasmid DNAs were extracted from the bacteria using the DNA preparation procedures of Birnboin and Doly, Nucleic Acid Res.7: 1515 (1979), and the double-stranded plasmid DNAs were submitted to DNA sequence determinations using Sequenase® (United States Biochemicals) and T7 and SP6 specific sequencing primers (Boehringer Mannheim) using the protocols recommended by the manufacturer. A unique DNA sequence representing a murine IgG2a heavy chain variable region was obtained, as was a kappa light chain variable region sequence.

To give the final appearance of a "veneered" murine light chain, several residues within a template composed of the human LEN framework, into which had been grafted the CDRs described for 1B4, were replaced by corresponding residues found in the murine 1B4 light chain framework. Replacement of the human LEN variable region residues with those unique to MAb 1B4 took place as follows. Eight oligodeoxynucleotides (Figure 5) were synthesized representing the primers necessary to generate by PCR amplification four DNA fragments. Incorporated into all but the terminal oligodeoxynucleotides were those sequences corresponding to the MAb 1B4 light chain variable region framework residues to be point mutated and at least 15 bases of 5'-terminal complementarity (see Figure 6). The appropriate primer pair (50 pmole each) was combined with 10 ng of a 1B4 CDR-grafted LEN framework-containing plasmid DNA, 2.5 units of Taq DNA polymerase, PCR reaction components and buffer, and twenty-five (25) cycles of PCR amplification ensued (cycle periods: 1', 94°C; 1', 55°C; 2' 72°C). The products of the four reactions, purified by agarose gel electrophoresis, were combined (10 ng of each DNA fragment) along with a terminal oligodeoxynucleotide primer pair (amplifier) (Figures 5 & 6), Taq DNA polymerase, PCR reaction components and buffer, and the subsequent recombined fragments were amplified, as described above, for twenty-five (see Figure 6). Following restriction endonuclease digestion with HindIII and XbaI the amplified DNA was purified from an agarose gel and subcloned into these same sites of an intermediate vector pSP72 (Promega) which contained the human kappa light chain constant region, obtained as follows. DNA (1μg) purified from a human B cell line (GM01018A; NIGMS Human Genetic Mutant Cell Repository, Institute for Medical Research, Camdan, N.J. 08103) was used as a template for the oligodeoxynucleotide primers described in Figure 4 to PCR amplify a 920 base pair fragment containing the splice acceptor for the human kappa light chain constant domain, the exon and a portion of its 3'-untranslated region (PCR primer pair choice was selected based on the kappa constant region sequence described by Hieter et al., Cell 22: 197-207 (1980). The PCR product was purified by agarose gel electrophoresis, digested with Bam H1 endonuclease, and subcloned into pSP72 (Promega) previously linearized with Bam H1.

The individual clones representing the pSP72 intermediate vector containing both the 1B4 "veneered" light chain variable region derived as described above, and the human kappa constant region, derived by PCR amplification of human DNA, were used to verify the DNA sequence of the "veneered" light chain variable region. The "veneered" heavy chain portion of the recombinant antibody was derived from a point mutated murine 1B4 heavy chain variable region fused to the human constant region of gamma 4 subtype obtained from a lambda library constructed by Flanagan and Rabbitts, Nature 300: 709-713 (1982).

The variable region of the "veneered" heavy chain was constructed from five DNA fragments representing a signal sequence, mutated portions of the murine 1B4 heavy chain variable region, and an intronic sequence (Figure 8). Oligodeoxynucleotide primer pairs (Figure 5) were synthesized representing the primers necessary to generate by PCR amplification these five DNA fragments from 10 ng of plasmid DNA template containing the murine 1B4 heavy chain variable region previously used to determine the murine 1B4 CDR and framework sequences. Amplification of the five fragments was performed as described above for the four

light chain variable region fragments. The agarose gel purified products were combined (10 ng of each product) with terminal primer pairs (Figure 5) and the PCR-generated *in vitro* recombined template was amplified using the standard procedure also described above for recombining the fragments comprising the "veneered" light chain variable region. Prior to subcloning into a Hind III and Bam HI digested expression vector this recombined product was similarly digested and agarose gel purified. DNA was obtained following growth of individual bacterial clones and submitted to DNA sequence determination using Sequenase® and T7 and SP6 specific sequencing primers in order to verify the sequence of the reconstructed variable region and its flanking domains.

The gamma 4 heavy chain constant region had been subcloned as a 6.7 Kb Hind III fragment derived from the plasmid pAT84 (Flanagan and Rabbitts, supra)into the Hind III site of the intermediate vector pSP72 (Promega). This plasmid was then used as the template DNA from which a shortened version of the gamma 4 constant region was obtained using the standard PCR amplification procedures described above and the primer pairs indicated in Figure 4. Eukaryotic expression vectors were constructed as described below such that the heavy chain immunoglobulin molecule was transcribed from a plasmid carrying the neomycin (G418) (Rothstein and Reznikoff, Cell 23: 191-199 [1981]) resistance marker, while the light chain immunoglobulin was transcribed from a plasmid carrying the hygromycin B resistance marker (Gritz and Davies, Gene 25: 179-188 [1983]). With the exception of the drug resistance portion of these plasmids they are identical.

The progenitor of the immunoglobulin expression vectors was the pD5 eukaryotic expression vector (Berkner and Sharp, Nucl. Acids Res. 13: 841-857 [1985]) which contained the origin of adenovirus replication, the SV40 enhancer domain, the adenovirus major late promoter, the adenovirus 2 tripartite leader, a 5' splice donor from the adenovirus third leader and a 3' splice acceptor derived from an immunoglobulin locus, a multiple cloning site, and the SV40 late polyadenylation signal (Figure 10). The origin of replication was removed by digestion with Eco R1 and Kpn I and replaced by two fragments representing the neo selectable marker gene (derived from plasmid pCMVIE-AK1-DHFR (Silberklang et al., Modern Approaches to Animal Cell Technology, Ed. Spier et al., Butterworth, U.K., [1987]) as an Eco R1/Bam H1 1.8 Kb fragment) and the Ig heavy chain enhancer (obtained as a PCR amplified fragment using standard procedures described above and human DNA as the template; the oligodeoxynucleotide primer pair is listed in Figure 4) following its digestion with Bgl II and Kpn I. The resultant expression vector was found to lack a small portion of the TK promoter responsible for the transcription of the neomycin gene. This was replaced by insertion into the Eco RI site of a 0.14 kb PCR amplified fragment derived from the CMVIE-AK1-DHFR DNA using the primer pair also listed in Figure 4. The resultant heavy chain expression vector was subsequently modified by removal of the indicated Hind III and Xba I sites. To convert this neomycin selectable vector into one expressing the hygromycin B selectable marker (Figure 11) the neomycin-resistance cassette was removed by digestion first with Eco R1 followed by DNA polymerase-directed fill in of the 5' overhang, then subsequent Sal I digestion. The 1.9 kb hygromycin B expression cassette [TK promoter and TK polyadenylation signal flanking the hygromycin B gene obtained from Gritz and Davies, Gene 25: 179-188(1983), as the 1.9 kb Bam H1 fragment in plasmid (pLG90)] was removed from the plasmid pAL-2 by Bam H1 digestion and subcloned into the Bam H1 site of the intermediate vector pSP72 (Promega). The hygromycin B cassette was removed from this vector by digestion with Sma I and Sal I and cloned into the expression vector linearized as described above to create a blunt end and Sal I end DNA fragment.

Expression of the 1B4 "veneered" kappa light chain was accomplished by transferring this cistron from its position within the pSP72 intermediate vector to the hygromycin B selectable eukaryotic expression vector (Figure 7). A 1.5 kb DNA fragment resulting from the endonuclease digestion of v1B4 VK/pSP72 intermediate vector with Spe I and Cla I was purified by agarose gel electro-phoresis and ligated into the expression vector which had previously been linearized, by digestion with the same two restriction enzymes and agarose gel purified.

The 1B4 "veneered" heavy chain eukaryotic expression vector was constructed in one step (Figure 9) from an existing vector previously constructed to express a chimaeric form of the 1B4 heavy chain. The "veneered" heavy chain variable region created by PCR amplification (Figure 8) was digested with Hind III and Bam H1. The agarose gel purified 0.8 kb fragment was ligated into the Hind III and Bam H1 sites of the pD5/IgH-Enhancer/Neo/1B4 VH-Short Human C-Gamma 4 expression vector following its endonuclease digestion with these two enzymes and subsequent purification by agarose gel electrophoresis (Figure 9). Transformants containing both variable and constant regions were identified. Plasmid DNAs were grown (Maniatis et al., supra) and purified for transfection into recipient mammalian cells (Maniatis et al., supra; Birbion and Doly, supra.).

Equal amounts (10µg) of the plasmids encoding the "veneered" IgG4 heavy chain and the "veneered" kappa light chain were transfected by standard calcium phosphate precipitation procedures into human 293 cells and african green monkey kidney CV-1P cells. The culture supernatant fluids were assayed by a trapping Elisa (described below) for the secretion of a human kappa light chain containing IgG4 immunoglobulin.

An Elisa was developed for the quantitation of the amounts of a 1B4 recombinant antibody expressed in conditioned mammalian cell growth medium. Immulon-2 (Dynatech Labs.) 96-well plates ere coated overnight with a 5µg/ml solution of mouse anti-human k chain constant domain monoclonal antibody (cat. #MC009, The Binding Site, Inc., San Diego, CA) in 0.1M $NaHCO_3$ buffer (pH 8.2) at 4°C, and blocked with 1% bovine serum (BSA) in 0.1M $NaHCO_3$ for 1h at 25°C. After this and all subsequent steps, washing was performed with phosphate buffered saline (PBS). The wells are then challenged with conditioned medium containing recombinant anti-CD18 antibody, or with predetermined quantities of human IgG4 purified by protein A Sepharose (Pharmacia Fine Chemicals) chromatography from human IgG4 myeloma serum (cat. # BP028,The Binding Site, Inc.). All samples are diluted in PBS containing 0.05% Tween-20. 100µl aliquots are incubated for 1h at 37°C in triplicate, and standard calibration curves are constructed using IgG4 concentrations ranging from 10 ng/ml to 100 ng/ml. Bound and fully assembled human IgG4 (either native or recombinant "veneered"1B4 human IgG4 constructs) are detected with 100µl aliquots of a 1:500 dilution of mouse anti-human IgG4 Fc monoclonal antibody conjugated to alkaline phosphatase (cat #05-3822, Zymed Laboratories, Inc.) in phosphate buffered saline (PBS) containing 1% BSA. After incubation for 1h at 37°C and subsequent washing, the quantities of bound conjugate are detected by incubating all samples with a 1 mg/ml solution of p-nitrophenyl phosphate in 0.1M 2,2'amino-methylpropanediol buffer, pH 10.3, for 30 min at 25°C. The adsorbance of the wells is determined with a UV Max ELISA plate reader (Molecular Devices) set at 405 nm. All supernatant fluids contain this immunoglobulin, though in various amounts. The antibody secreted by the transfected 293 cells is concentrated by protein A chromatography and the concentrations of the recombinant human "veneered" anti-CD18 antibody determined by the trapping Elisa described above, is used to compete with the binding of radiolabeled murine 1B4 to the CD18 ligand on the surface of activated human PMNs. Affinities of various anti-CD18 antibody constructs are determined using a competitive ^{125}I-m1B4 soluble binding assay with stimulated human polymorphonuclear leukocytes (PMNs). Purified murine anti-CD18 monoclonal antibody (50 ug; m1B4) is iodinated using chloramine-T (Hunter, W.M. and Greenwood, F.C., Nature 194: 495-496, 1962), and the radiolabeled antibody purified using a Bio-Sil TSK250 (Biorad, Richmond, CA) gel filtration HPLC column (which fractionates proteins in the range of 1-300 x 10^3 daltons) equilibrated in 0.1M phosphate buffer, pH 7.0. Effluent radioactivity is monitored with an in-line detector (Beckman Model 170; Beckman, Fullerton,CA) and total protein measured at OD_{280} with a Kratos Spectroflow 757 detector (Kratos, Mawah, N.J.). A single ^{125}I-m1B4 peak composed of coincident OD_{280} and radioactivity tracings characteristically elutes 6 minutes, 30 seconds following sample injection. Specific activity of the product is generally about 10µCi/µg protein, and 97-99% of the counts are precipitable with 10% trichloroacetic acid. The binding of this radiolabeled antibody is assessed on human PMNs purified on a discontinuous Ficoll/Hypaque gradient (English, D. and Anderson, B.R., J. Immunol. Methods 5: 249-255, 1974) and activated with 100 ng/ml phorbol myristate for 20 minutes at 37°C (Lo, et al., J. Exp. Med. 169: 1779-1793, 1989). To determine the avidity of antibodies for CD18 molecules on the PMN surface, about 1 x 10^5 activated PMNs are incubated in a buffer such as Hanks balanced salt solution containing 20 mM Hepes (pH 7.2), 0.14 units aprotinin (Sigma Chemical Co.) and 2% human serum albumin (binding buffer) containing 1.3 ng ^{125}I-m1B4 (2.8 x 10^{-11}M) in the presence of increasing concentrations of unlabeled m1B4 antibody (10^{-7} to 10^{-15}M) in a 300 ul reaction volume for about 1 h at about 4°C with constant agitation. Cell bound 1B4 is separated from the unbound antibody by centrifugation through a 0.5M sucrose cushion (4,800 x·g, 3 minutes); the tubes are frozen on dry ice, and the tips cut off and counted with an LKB gamma counter. The IC_{50} of the anti-CD18 antibody for the inhibition of ^{125}I-m1B4 antibody binding is calculated using a four parameter fitter program (Rodbard, D, Munson, P.J., and DeLean, In, "Radioimmunoassay and Related Procedures in Medicine", International Atomic Energy Agency, Vienna, vol I,469-504, 1978). The affinity of the "veneered" anti-CD18 antibody for the CD18 ligand is determined in a similar manner using murine ^{125}I-m1B4 antibody and increasing quantities, as determined by the trapping Elisa, of unlabeled "veneered" anti-CD18 antibody. The results of the binding assays are shown in Figure 13 and indicate that the avidity of the "veneered" heavy chain and light chain recombinant 1B4 antibody is equivalent to that of the murine 1B4 monoclonal antibody.

The "veneered" heavy and light chain expression vectors were co-transfected into CV1P mon-

key kidney cells using 20 µg of each plasmid to prepare 2 mL of the calcium phosphate precipitated solution. One mL was placed in the medium overlaying each 100 mm dish of CV1P cells. After 4 hr at 37°C the medium was replaced with 1 mL of 15% glycerol in 1 x HBS (Hepes buffered salt). Following the 3 min glycerol shock, 10 mL of PBS as added, the cell monolayers were aspirated, washed once with 10 mL of PBS, and re-fed with fresh medium (DMEM + 10% heat inactivated new born calf serum) containing 200 µg of hygromycin B and 800 µg of G418 per mL. Cloning cylinders (Fishney, In, Culture of Animal Cells, Alan R. Liss, Inc. New York, 1983) were used to isolate individual colonies prior to their expansion and subsequent assay for productivity. Two clones, #11 and #48, were found to express sufficient amounts of v1B4 to warrant their expansion and ultimete accessioning.

Claims

1. A method for identifying differences in mammalian speciee specific eurface emino acid residues on en immunoglobulin comprising:
   e. compering the framework amino ecids of a varieble domein of e first mammalien species with the variable domains of a second mammallen species;
   b. determining the subgroups of the second memmellen species to which the first mammalian species most closely corresponds;
   c. determining the second mammelian species eequence which is most similer to the first mammalian species sequence;
   d. identifying amino acid residues of the first memmelian species which differ from the emino ecid residues of the second mammallen species, with said amino acids being mostly exposed or completely exposed on the immunoglobulin surface;
   e. identifying only those amino acid residues which are not within e complementarity-determining region or are not directly adjacent to a complementarity-determining region.

2. The method of claim 1 wherein the first mammalian species is mouse.

3. The method of claim 1 wherein the second mammalian species is human.

4. A method for converting an immunoglobulin having the immunogenicity of a first mammalian species to an antibody having the immunogenicity of a second mammalian species comprising:
   a. replacing the amino acid residues in a first mammalian species framework which differ from the amino acid residues of a second mammalian species with the corresponding amino acid residues from the most similar second mammalian species es identified by the method of claim 1.

5. The method of claim 2 wherein the first mammalian species is mouse.

6. The method of claim 2 wherein the second mammalian species is human.

7. A method comprising:
   a. preparing a DNA sequence encoding e veneered immunoglobulin having specificity for a known antigen wherein the surface amino acid residues of a first mammalian species which differ from the surface emino acid residuee of e second mammalian species are repleced with the corresponding amino acids residues form the most similar second second memmelien species sequence es identified by the method of claim 1;
   b. inserting the sequence into a replicable expression vector operably linked to e suitable promoter compatible with e host cell;
   c. transforming the host cell with the vector of b;
   d. culturing the host cell; and
   e. recovering the veneered immunoglobulin from the host cell culture.

8. The method of claim 7 wherein the first mammalian species is mouse.

9. The method of claim 7 wherein the second mammalian species is human.

10. A composition comprising e veneered immunoglobulin having e specificity for e known antigen.

11. A DNA sequence encoding veneered 1B4 antibody.

12. A veneered murine 1B4 antibody exhibiting the antigenicity of human antibody of fragments thereof.

Fig. 1a

Position	Fractional Accessibility KOL Residue	Exposure	J539 Residue	Exposure	Residues In Subgroup I	II	III
1	E	1.00 Ex	E	1.00 Ex	Q	Q	E
2	V	0.23 mB	V	0.37 mB	V	V	V M
3	Q	0.82 Ex	K	0.82 Ex	Q	T Q	Q
4	L	0.00 Bu	L	0.10 Bu	L	L	L
5	V	0.87 Ex	L	1.00 Ex	V	RQKT	VL
6	Q	0.00 Bu	E	0.09 Bu	Q	E	E
7	S	0.94 Ex	S	0.94 Ex	S	S	S
8	G	1.00 Ex	G	1.00 Ex	G	G	G
9	G	0.00 Bu	G	0.00 Bu	A	P	G
10	G	1.00 Ex	G	1.00 Ex	E	AGT	GA
11	V	0.90 Ex	L	0.81 Ex	V	L	LF
12	V	0.25 mB	V	0.25 mB	K	V	V
13	Q	0.71 mE	Q	0.87 Ex	K	K	Q
14	P	0.59 PB	P	0.64 mE	P	P	P
15	G	1.00 Ex	G	1.00 Ex	G	TS	G
16	R	0.73 mE	G	1.00 Ex	SA	EQ	G
17	S	0.66 mE	S	0.75 mE	S	T	S
18	L	0.28 mB	L	0.26mB	V	L	L
19	R	0.66 mE	K	0.75 mE	RK	TS	RK
20	L	0.00 Bu	C	0.00 Bu	V	L	L
21	S	0.71 mE	S	0.82 Ex	S	T	S
22	C	0.00 Bu	C	0.00 Bu	C	C	C
23	S	1.00 Ex	A	1.00 Ex	K	T	A
24	S	0.00 Bu	A	0.00 Bu	ATV	FV	A
25	S	0.87 Ex	S	1.00 Ex	S	S	S
26	G	1.00 Ex	G	1.00 Ex	G	G	G
27	F	0.10 Bu	F	0.10 Bu	GYD	FLG	F
28	I	0.85 Ex	D	0.72 mE	T	S	TN
29	F	0.00 Bu	F	0.00 Bu	F	LI	F
30	S	0.74 mE	S	0.83 Ex	SNVI	S	S
36	W	0.00 Bu	W	0.00 Bu	W	W	W
37	V	0.00 Bu	V	0.00 Bu	V	I	V
38	R	0.10 Bu	R	0.31 mB	R	R	R
39	Q	0.15 Bu	Q	0.28 mB	Q	Q	Q
40	A	0.95 Ex	A	0.75 mE	A	P	A
41	P	0.90 Ex	P	0.73 mE	P	P	PS
42	G	1.00 Ex	G	1.00 Ex	G	G	G
43	K	0.86 Ex	K	0.86 Ex	QRKH	KR	K
44	G	1.00 Ex	G	1.00 Ex	G	AG	GS
45	L	0.00 Bu	L	0.00 Bu	L	L	L

Fig. 1b

	Fractional Accessibility				Residues in Subgroup					
Position	KOL		J539		I		II		III	
	Residue	Exposure	Residue	Exposure						
46	E	0.75 mE	E	0.73 mE	E		E		E	
47	W	0.10 Bu	W	0.04 Bu	W		W		W	
48	V	0.00 Bu	I	0.00 Bu	MV		LI		V	
49	A	0.00 Bu	G	0.00 Bu	G		AG		GSA	
66	R	0.36 mB	K	0.51 pB	R		R		R	
67	F	0.00 Bu	F	0.00 Bu	V		LV		F	
68	T	0.87 Ex	I	0.88 Ex	T		T		T	
69	I	0.00 Bu	I	0.00 Bu	VMI		IV		I	
70	S	0.78 mE	S	0.79 mE	TS		ST		S	
71	R	0.11 Bu	R	0.00 Bu	RLA		KV		R	
72	N	0.61 mE	D	0.55 pB	DK		D		DN	
73	D	0.44 pB	N	0.43 pB	PETAS		T		DN	
74	S	0.85 Ex	A	0.97 Ex	S		S		S	
75	K	0.88 Ex	K	0.77 mE	TF		KR		K	
76	N	0.69 mE	N	0.68 mE	NST		N		N	
77	T	0.41 pB	S	0.33 mB	TQ		Q		T	
78	L	0.00 Bu	L	0.00 Bu	AV		VF		LA	
79	F	0.45 pB	Y	0.35 mB	Y		VS		YF	
80	L	0.00 Bu	L	0.00 Bu	M		L		L	
81	Q	0.53 pB	Q	0.69 mE	E		TKSIN		Q	
82	M	0.00 Bu	M	0.00 Bu	L		ML		M	
82a	D	0.73 mE	S	0.58 pB	SVRT		TSNIR		ND	
82b	S	0.98 Ex	K	0.96 Ex	S		NS		S	
82c	L	0.00 Bu	V	0.00 Bu	L		VM		L	
83	R	0.73 mE	R	0.83 Ex	RFI		DT		RE	
84	P	0.75 mE	S	0.90 Ex	S		PA		PA	
85	E	0.82 Ex	E	0.90 Ex	E		VA		ED	
86	D	0.00 Bu	D	0.11 Bu	D		D		D	
87	T	0.54 pB	T	0.47 pB	T		T		T	
88	G	1.00 Ex	A	0.00 Bu	A		A		A	
89	V	0.58 PB	L	0.63 mE	V		TV		VL	
90	Y	0.00 Bu	Y	0.00 Bu	Y		Y		Y	
91	F	0.00 Bu	Y	0.08 Bu	Y		Y		Y	
92	C	0.00 Bu	C	0.00 Bu	C		C		C	
93	A	0.00 Bu	A	0.00 Bu	A		A		AT	
94	R	0.17 Bu	R	0.15 Bu	R		RH		RP	
					JHI	JH2	JH3	JH4	JH5	JH6
103	W	0.09 Bu	W	0.07 Bu	W	W	W	W	W	W
104	G	0.00 Bu	G	1.00 Ex	G	G	G	G	G	G

Fig. 1c

Position	Fractional Accessibility KOL Residue	KOL Exposure	J539 Residue	J539 Exposure	Residues In Subgroup I JHI	II JH2	II JH3	II JH4	III JH5	III JH6
105	Q	0.93 Ex	Q	0.99 Ex	Q	R	Q	Q	Q	Q
106	G	0.00 Bu	G	0.00 Bu	G	G	G	G	G	G
107	T	0.22 mB	T	0.26 mB	T	T	T	T	T	T
108	P	0.99 Ex	L	0.67 mE	L	L	M	L	L	T
109	V	0.00 Bu	V	0.00 Bu	V	V	V	V	V	V
110	T	0.76 mE	T	0.69 mE	T	T	T	T	T	T
111	V	0.00 Bu	V	0.00 Bu	V	V	V	V	V	V
112	S	0.98 Ex	S	0.74 mE	S	S	S	S	S	S
113	S	0.94 Ex	A	0.84 Ex	S	S	S	S	S	S

Fig. 2a

Position	Residue	Exposure	I	II	III	IV	V	VI
			Residues In Subgroup					
1	Q	1.00 Ex	Q	Q	SF	-	Q	ND
2	S	1.00 Ex	S	S	Y	S	S	F
3	V	0.77 mE	V	A	E	E	A	M
4	L	0.00 Bu	L	L	L	L	L	L
5	T	0.92 Ex	T	T	TK	T	T	T
6	Q	0.00 Bu	Q	Q	Q	Q	Q	Q
7	P	0.62 mE	P	P	P	D	P	P
8	P	1.00 Ex	P	ARP	P	P	P	H
9	S	1.00 Ex	S	S	S	A	S	S
10	-	-	-	-	-	-	-	-
11	A	0.34 mB	AV	V	V	V	A	V
12	S	0.71 mE	S	S	S	S	S	S
13	G	1.00 Ex	GA	G	VL	V	G	E
14	T	0.73 mE	TA	S	SA	A	S	S
15	P	0.75 mE	P	P	PA	L	PL	P
16	G	1.00 Ex	G	G	G	G	G	G
17	Q	0.69 mE	Q	Q	Q	Q	Q	K
18	R	0.79 mE	R	S	T	T	S	T
19	V	0.21 mB	V	IV	A	V	V	V
20	T	0.62 ME	T	T	RM	R	T	T
21	I	0.00 Bu	I	I	I	I	I	IFM
22	S	0.92 Ex	S	S	T	T	S	S
23	C	0.00 Bu	C	C	C	C	C	C
35	W	0.00 Bu	W	W	W	W	W	W
36	Y	0.00 Bu	Y	YF	Y	Y	Y	Y
37	Q	0.46 pB	Q	Q	Q	Q	Q	Q
38	Q	0.00 Bu	QH	Q	QE	Q	Q	Q
39	L	0.75 mE	LV	H	KR	K	H	R
40	P	0.91 Ex	P	P	PS	P	PA	P
41	G	1.00 EX	G	G	G	G	G	G
42	M	0.74 mE	T	K	QR	Q	RK	SRG
43	A	0.62 mE	A	A	A	A	A	A
44	P	0.00 Bu	P	P	P	P	P	P
45	K	0.95 Ex	K	K	V	L	K	T
46	L	0.23 mB	L	L	MLP	L	LV	T
47	L	0.15 Bu	L	MIL	V	V	VI	V
48	I	0.00 Bu	I	I	IV	I	I	I
49	Y	0.39 mB	Y	YF	Y	Y	FY	Y
57	G	1.00 Ex	G	G	GE	G	G	G
58	V	0.14 Bu	VI	VI	IV	I	V	V
59	P	0.70 mE	P	SP	P	P	P	P
60	D	0.95 Ex	D	DNL	EQA	D	D	D
61	R	0.31 mB	R	R	R	R	R	R

Fig. 2b

Position	Residue	Exposure	I	II	III	IV	V	VI
			Residues In Subgroup					
62	F	0.12 Bu	F	F	F	F	F	F
63	S	0.85 Ex	S	S	S	S	S	S
64	G	0.00 Bu	G A	G	G S	G	G	G
65	S	1.00 Ex	S	S	S Y	S	S	S
66	K	0.41 pB	K	K	T S N	S	K	I F*
67	S	1.00 Ex	S	S	S	S	S	S
68	G	1.00 Ex	G	G	G	G	D G	S
69	A	0.71 mE	T	N	T N	H	N	N
70	S	1.00 Ex	S	T	T K S	T	T	S
71	A	0.00 Bu	A	A	A V	A	A	A
72	S	1.00 Ex	S T	S	T I	S	S	S
73	L	0.00 Bu	L	L	L	L	L	L
74	A	0.74 mE	A	T	T	T	T	T
75	I	0.00 Bu	I	I	I	I	V	I
76	G	1.00 Ex	S T	S	S N	T	S	S
77	G	1.00 Ex	G	G	G R	G	G	G
78	L	0.00 Bu	L	L	V A	A	L	L
79	Q	0.76 mE	Q R	Q	Q E	Q	R Q	K Q T
80	S	1.00 Ex	S T	A	A V	A	A	T
81	E	0.78 mE	E G	E	E G	E	E	E
82	D	0.09 Bu	D	D	D	D	D	D
83	E	0.64 mE	E	E	E	E	E	E
84	T	0.34 mB	A	A	A	A	A	A
85	D	0.30 mB	D	D	D	D	D	D
86	Y	0.00 Bu	Y	Y	Y	Y	Y	Y
87	Y	0.16 Bu	Y	Y	Y F	Y	Y	Y
88	C	0.00 Bu	C	C	C	C	C	C
			JL-1	JL-2	JL-3	JL-4	JL-5	
98	F	0.04 Bu	F	F	F	F	F	
99	G	0.00 Bu	G	G	G	G	G	
100	T	0.59 pB	T	G	G	S	S	
101	G	1.00 Ex	G	G	G	G	G	
102	T	0.00 Bu	T	T	T	T	T	
103	K	0.82 Ex	K	K	K	Q	Q	
104	V	0.00 Bu	V	L	L	L	L	
105	T	0.86 Ex	T	T	T	T	T	
106	V	0.19 Bu	V	V	V	V	V	
106a	L	0.70 mE	L	L	L	L	L	
107	G	1.00 Ex	G	G	G	S	G	

* additional residues after position 66:
66a D
66b S R D

Fig. 3a

Position	Residue	Exposure	Residues in Subgroup I	II	III	IV
1	E	0.99 Ex	D	D	E	D
2	I	0.16 Bu	I	I	I	I
3	V	0.87 Ex	Q	V	V	V
4	L	0.00 Bu	M	M	L	M
5	T	0.80 mE	T	T	T	T
6	Q	0.00 Bu	Q	Q	Q	Q
7	S	0.89 Ex	S	S	S	S
8	P	0.67 mE	P	P	P	P
9	A	1.00 Ex	S	L	G	D N
10	I	0.94 Ex	S	S	T	S
11	T	0.30 mB	L	L	L	L
12	A	0.59 pB	S	P	S	A
13	A	0.00 Bu	A	V	L	V
14	S	0.78 mE	S	T	S	S
15	L	0.79 mE	V	P	P	L
16	G	1.00 Ex	G	G	G	G
17	Q	0.64 mE	D	E	E	E
18	K	0.74 mE	R	P	R	R
19	V	0.22 mB	V	A	A	A
20	T	0.65 mE	T	S	T	T
21	I	0.00 Bu	I	I	L	I
22	T	0.69 mE	T	S	S	N
23	C	0.00 Bu	C	C	C	C
35	W	0.00 Bu	W	W	W	W
36	Y	0.00 Bu	Y	Y	Y	Y
37	Q	0.14 Bu	Q	L	Q	Q
38	Q	0.24 mB	Q	Q	Q	Q
39	K	0.69 mE	K	K	K	K
40	S	1.00 Ex	P	P	P	P
41	G	1.00 Ex	G	G	G	G
42	T	0.90 Ex	K	Q	Q	Q
43	S	0.30 mB	A	S	A	P
44	P	0.00 Bu	P	P	P	P
45	K	0.90 Ex	K	QER	R	K
46	P	0.43 pB	L	L	L	L
47	W	0.16 Bu	L	L	L	L
48	I	0.00 Bu	I	I	I	I
49	Y	0.42 pB	Y	Y	Y	Y
57	G	1.00 Ex	G	G	G	G
58	V	0.13 Bu	V	V	I	V
59	P	0.61 mE	P	P	P	P
60	A	1.00 Ex	S	D	D	D
61	R	0.36 mB	R	R	R	R
62	F	0.00 Bu	F	F	F	F
63	S	0.94 Ex	S	S	S	S

Fig. 4

Mouse Light Chain Variable Region

5' upstream primer - FR1 of variable region

5'- TCT CGG ATC CGA (CT)AT (TC)GT G(AC)T (GC)AC CCA (GA) -3'

Bam H1

3' downstream primer - kappa constant region

5'- TCT CAA GCT TTG GTG GCA AGA T(GA)G ATA CAG TTG GTG CAG C -3'

Hind III

Mouse Heavy Chain Variable Region

5' upstream primer - FR1 of variable region

i) 5'- TTC TGG ATC C(CG)A GGT (GCT)CA (AG)CT G(AC)A G(GC)A GTC (TA)GG -3'

Bam H1

ii) 5'- TTC TGG ATC C(CG)A GGT (GCT)AA GCT GGT G(GC)A GTC (TA)GG -3'

Bam H1

3' downstream primer - IgG2a CH1 region

5'- TCT CAA GCT TAC CGA TGG (GA)GC TGT TGT TTT GGC -3'

Hind III

SHORTEN VERSION OF THE IgG4 HEAVY CHAIN CONSTANT REGION

5'- ATT TGG ATC C TC TAG A CA TCG CGG ATA GAC AAG AAC -3'

Bam H1 Xba I

5'- AAT AAT GCG GCC GC A TCG AT G AGC TCA AGT ATG TAG ACG GGG TAC G -3'

Not I Cla I Sac I

TK PROMOTER FRAGMENT

5'- TAT AGA ATT C GG TAC CCT TCA TCC CCG TGG CCC G -3'

Eco R1 Kpn I

5'- TGC GTG TTC GAA TTC GCC -3'

Eco R1

Ig H ENHANCER

5'- TTT TAG ATC T GT CGA CAG ATG GCC GAT CAG AAC CAG -3'

Bgl II Sal I

5'- TTG GTC GAC GGT ACC AAT ACA TTT TAG AAG TCG AT -3'

Sal I Kpn I

HUMAN KAPPA CONSTANT REGION

5'- TCT CGG ATC CTC TAG AAG AAT GGC TGC AAA GAG C -3'

5'- TCT CGC TAG CGG ATC CTT GCA GAG GAT GAT AGG G -3'

Fig. 5

Oligodeoxynucleotides for PCR Amplification of the LEN Light Chain Variable Region

S1 5'- CAT TCG CTT ACC AGA TCT AAG CTT ACT AGT GAG ATC ACA GTT CTC TCT AC -3'
V9 5'- TGG CTC TGC AGC TGA TGG TG -3'

V10 5'- CAC CAT CAG CTG CAG AGC CA -3'
V11 5'- CTG TCT GGG ATC CCA GAT TC -3'

V12 5'- GAA TCT GGG ATC CCA GAC AG -3'
V13 5'- GTT GCA ACA TCT TCA GCC TCC ACG CTG CTG ATG -3'

V14 5'- GTG GAG GCT GAA GAT GTT GCA ACT TAT TAC TG -3'
I3 5'- GAA TGT GCC TAC TTT CTA GAG GAT CCA ACT GAG GAA GCA AAG -3'

A1 5'- CAT TCG CTT ACC AGA TCT -3'
A2 5'- GAA TGT GCC TAC TTT CTA G -3'

Oligodeoxynucleotides for PCR Amplification of the m1B4 Heavy Chain Variable Region

V1 5'- CCC TCC AGG CTT CAC TAA GTC TCC CCC -3'

V2 5'- TTA GTG AAG CCT GGA GGG TCC CTG AAA CTC -3'
V3 5'- GCC CCT TCC CAG GAG CTT GGC GAA CCC AAG ACA TG -3'

V4 5'- AAG CTC CTG GGA AGG GGC TGG AGT TGG TCG CAG CC -3'
V5 5'- TGT TCA TTT GTA GGT ACA GGG TGT TCT TGG AAT TGT CTC TGG AGA TGG TG -3'

V6 5'- TGT ACC TAC AAA TGA ACA GTC TGA GGG CTG AGG ACA CAG CCT TGT ATT -3'
V7 5'- CTG TGA GAA GGG TGC CTT GGC CCC AGT AG -3'

V8 5'- AAG GCA CCC TTC TCA CAG TCT CCT CAG GTG -3'
I2 5'- GAA TGT GCC TAC TTA AGC TTT CTA GAG GAT CCT ATA AAT CTC TGG CCA TG -3'

S1, A1, and A2, as above

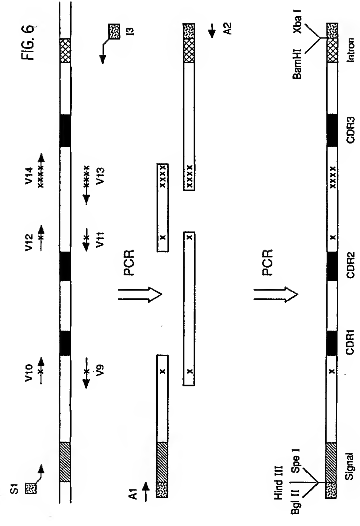

S1
V10
V9
V12
V11
V14
V13
I3
PCR
A1
A2
PCR
Bgl II
Hind III
Spe I
BamHI
Xba I
Signal
CDR1
CDR2
CDR3
Intron

FIG. 6

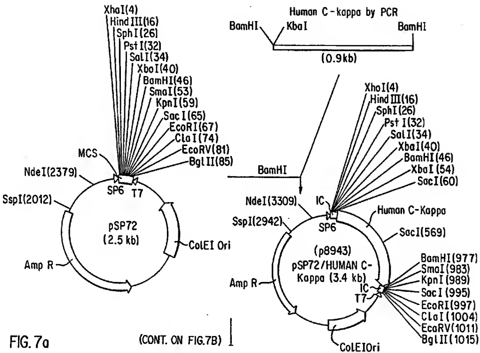
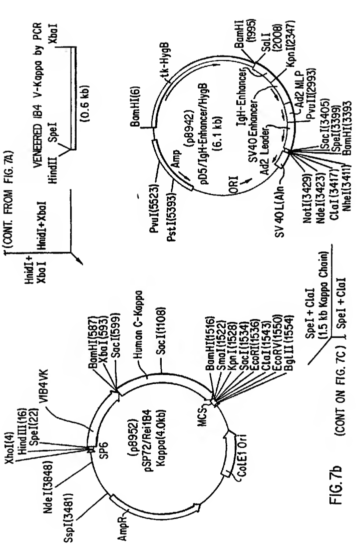


FIG. 7a

(CONT. ON FIG.7B)



(CONT. FROM FIG. 7A)
HnidI+ XbaI
HnidI+XbaI
HindII
SpeI
VENEERED IB4 V-Kappa by PCR
XbaI
(0.6 kb)
BamHI(6)
tk-HygB
BamHI (1995)
SalI (2008)
KpnI(2347)
IgH-Enhancer
IgH-Enhancer
SV40 Enhancer
Ad2 Leader
Ad2 MLP
PvuII(2993)
SacI(3405)
SpeI(3399)
BamHI(3393)
(p8942)
pD5/IgH-Enhancer/HygB
(6.1 kb)
Amp
ORI
PvuI(5523)
PstI(5393)
SV 40 L(A)n
NotI(3429)
NdeI(3423)
ClaI(3417)
NheI(3411)
SpeI + ClaI
(1.5 kb Kappa Chain)
SpeI + ClaI
XhoI(4)
HindIII(16)
SpeI(22)
VIB4VK
BamHI(587)
XbaI(593)
SacI(599)
Human C-Kappa
SacI(1108)
NdeI(3848)
SspI(3481)
SP6
(p8952)
pSP72/Rei1B4
Kappa(4.0kb)
MCS
BamHI(1516)
SmaI(1522)
KpnI(1528)
SacI(1534)
EcoRI(1536)
ClaI(1543)
EcoRV(1550)
BglII(1554)
AmpR
ColE1 Ori
(CONT ON FIG. 7C)

FIG. 7b

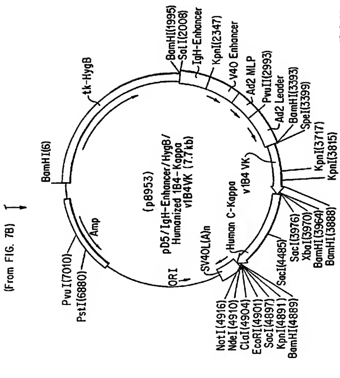
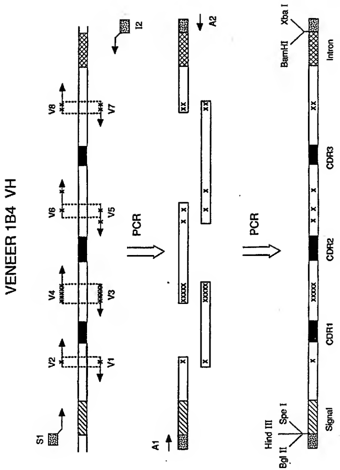

(From FIG. 7B)
BamHI(6)
tk-HygB
PvuI(7010)
PstI(6880)
Amp
ORI
(p8953)
pD5/IgH-Enhancer/HygB/
Humanized 1B4-Kappa
v1B4VK (7.7kb)
Human C-Kappa
v1B4 VK
SV40L(A)n
BamHI(1995)
SalI(2008)
IgH-Enhancer
KpnI(2347)
V40 Enhancer
Ad2 MLP
PvuII(2993)
Ad2 Leader
BamHI(3393)
SpeI(3399)
KpnI(3717)
KpnI(3815)
SacI(3976)
XbaI(3970)
BamHI(3964)
BamHI(3888)
SacI(4485)
NotI(4916)
NdeI(4910)
ClaI(4904)
EcoRI(4901)
SacI(4897)
KpnI(4891)
BamHI(4889)

FIG. 7c

Fig. 8


VENEER 1B4 VH
S1
V2
V1
V4
V3
V6
V5
V8
V7
I2
PCR
A1
A2
PCR
Bgl II
Hind III
Spe I
Signal
CDR1
CDR2
CDR3
BamHI
Xba I
Intron

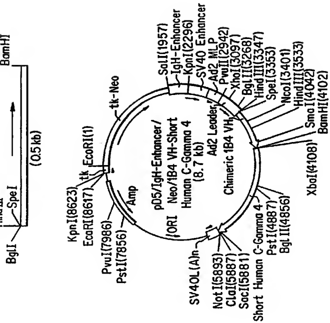

VENEERED 1B4 VH BY PCR
BglI
HindIII
SpeI
BamHI
(0.5 kb)
pD5/IgH-Enhancer/
Neo/1B4 VH-Short
Human C-Gamma 4
(8.7 kb)
Ad2 Leader
Chimeric 1B4 VH
tk-Neo
SalI(1957)
IgH-Enhancer
KpnI(2296)
SV40 Enhancer
Ad2 MLP
PvuII(2942)
XhoI(3097)
BglII(3268)
HindIII(3347)
SpeI(3353)
NcoI(3401)
HindIII(3533)
SmaI(4042)
BamHI(4102)
XbaI(4108)
Short Human C-Gamma 4
PstI(4887)
BglII(4856)
NotI(5893)
ClaI(5887)
SacI(5881)
SV40L(A)n
ORI
Amp
PvuI(7986)
PstI(7856)
KpnI(8623)
EcoRI(8617)
tk
EcoRI(1)

FIG. 9a

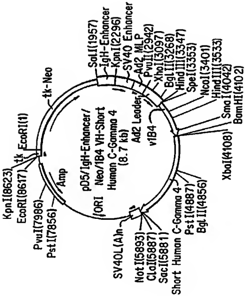

pD5/IgH-Enhancer/
Neo/1B4 VH-Short
Human C-Gamma 4
(8.7 kb)
SalI(1957)
IgH-Enhancer
KpnI(2296)
SV40 Enhancer
Ad2 MLP
PvuII(2942)
XhoI(3097)
BglII(3268)
HindIII(3347)
SpeI(3353)
NcoI(3401)
HindIII(3533)
SmaI(4042)
BamHI(4102)
XbaI(4108)
BglII(4856)
PstI(4887)
Short Human C-Gamma 4
SacI(5881)
ClaI(5887)
NotI(5893)
SV40L(A)n
ORI
PstI(7856)
PvuI(7986)
EcoRI(8617)
KpnI(8623)
tk
EcoRI(1)
tk-Neo
Amp
Ad2 Leader
v1B4

FIG. 9b

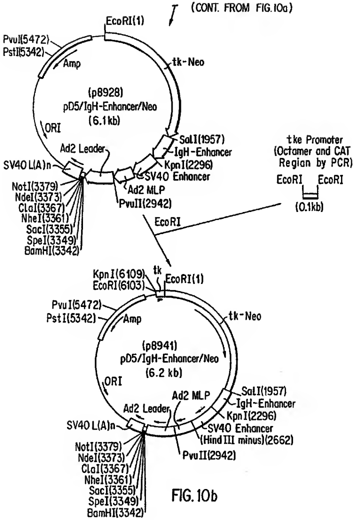

(CONT. FROM FIG.10a)
EcoRI(1)
PvuI(5472)
PstI(5342)
Amp
tk-Neo
(p8928)
pD5/IgH-Enhancer/Neo
(6.1 kb)
ORI
SalI(1957)
IgH-Enhancer
KpnI(2296)
SV40 Enhancer
Ad2 MLP
PvuII(2942)
Ad2 Leader
SV40 L(A)n
NotI(3379)
NdeI(3373)
ClaI(3367)
NheI(3361)
SacI(3355)
SpeI(3349)
BamHI(3342)
tke Promoter
(Octamer and CAT
Region by PCR)
EcoRI EcoRI
(0.1kb)
EcoRI
KpnI(6109)
EcoRI(6103)
tk
EcoRI(1)
PvuI(5472)
PstI(5342)
Amp
tk-Neo
(p8941)
pD5/IgH-Enhancer/Neo
(6.2 kb)
ORI
Ad2 MLP
SalI(1957)
IgH-Enhancer
KpnI(2296)
SV40 Enhancer
(Hind III minus)(2662)
PvuII(2942)
Ad2 Leader
SV40 L(A)n
NotI(3379)
NdeI(3373)
ClaI(3367)
NheI(3361)
SacI(3355)
SpeI(3349)
BamHI(3342)

FIG. 10b

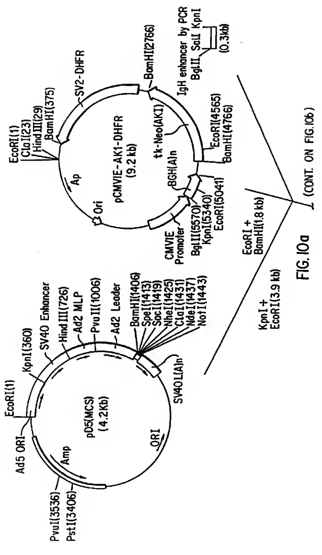
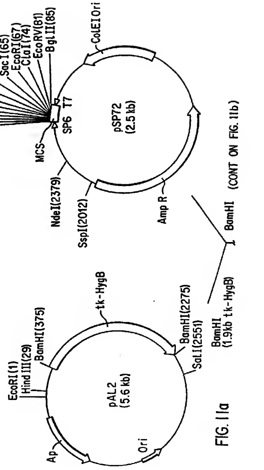

Ad5 ORI
EcoRI(1)
KpnI(360)
SV40 Enhancer
Hind III(726)
Ad2 MLP
Pvu II(1006)
Ad2 Leader
BamHI(1406)
SpeI(1413)
SacI(1419)
NheI(1425)
ClaI(1431)
NdeI(1437)
NotI(1443)
SV40 L(A)n
ORI
Amp
pD5(MCS)
(4.2Kb)
PvuI(3536)
PstI(3406)
KpnI+
EcoRI(3.9 kb)
EcoRI(1)
ClaI(23)
Hind III(29)
BamHI(375)
SV2-DHFR
BamHI(2766)
tk-Neo(AKI)
EcoRI(4565)
BamHI(4766)
EcoRI(5041)
KpnI(5340)
BglII(5570)
CMVIE
Promoter
BGH(A)n
Ori
Ap
pCMVIE-AK1-DHFR
(9.2 kb)
EcoRI +
BamHI(1.8 kb)
IgH enhancer by PCR
BglII SalI KpnI
(0.3kb)
(CONT. ON FIG.10b)

FIG. 10a



(CONT ON FIG. 11b)

FIG. 11a

(CONT. FROM FIG. 11a)

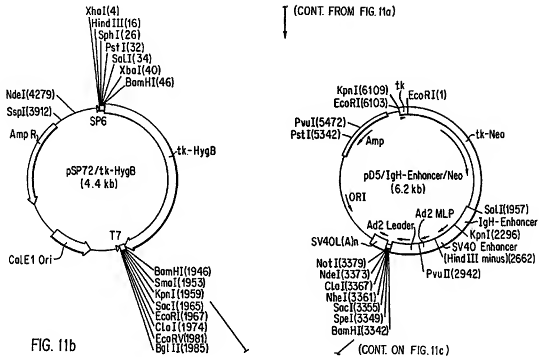


FIG. 11b

(CONT. ON FIG. 11c)

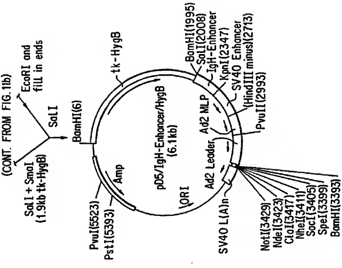

(CONT. FROM FIG. 11b)
SalI + SmaI
(1.9kb tk-HygB)
EcoRI and
fill in ends
SalI
BamHI(6)
tk-HygB
Amp
PvuI(5523)
PstI(5393)
pD5/IgH-Enhancer/HygB
(6.1kb)
ORI
Ad2 Leader
Ad2 MLP
SV40 L(A)n
BamHI(1995)
SalI(2008)
IgH-Enhancer
KpnI(2347)
SV40 Enhancer
(HindIII minus)(2713)
PvuII(2993)
NotI(3429)
NdeI(3423)
ClaI(3417)
NheI(3411)
SacI(3405)
SpeI(3399)
BamHI(3393)

FIG. 11c

Fig. 12

HEAVY AND LIGHT CHAIN VARIABLE REGION FRAMEWORKS

Heavy Chain

```
                  √                              √
v1B4: DVKLVESGGDLVKPGGSLKLSCAASGFTFS [DYYMS] WVRQAP
m1B4: DVKLVESGGDLVKLGGSLKLSCAASGFTFS [DYYMS] WVRQTP
 Gal: EVQLVESGGDLVQPGRSLRLSCAASGFTFS [BLGMT] WVRQAP

      √ √                                  √
      GKGLELVA [AIDNDGGSISYPDTVKG] RFTISRDNSKNTLYLQM
      EKRLELVA [AIDNDGGSISYPDTVKG] RFTISRDNAKNTLYLQM
      GKGLEWVA [NIKZBGSZZBYVDSVKG] RFTISRDNAKNSLYLQM

      √   √                               √
      NSLRAEDTALYYCAR [-QGRLRRDYFDY] WGQGTLLTVSS...
      SSLRSEDTALYYCAR [-QGRLRRDYFDY] WGQGTTLTVSS...
      NSLRVEDTALYYCAR [-----GWGGGD-] WGQGTLVTVST...
```

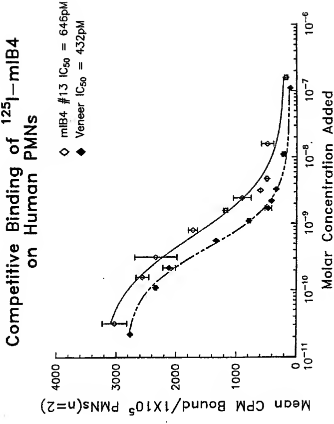
Light Chain

```
         √    √      √
v1B4: DIVMTQSSNSLAVSLGERATISC [RASESVDSYGNSFMH--] WY
m1B4: DIVLTQSPASLAVSLGQRATISC [RASESVDSYGNSFMH--] WY
 Len: DIVMTQSSNSLAVSLGERATINC [KSSQSVLYSSNSKNYLA] WY

                                 √       √       √√
      QQKPGQPPKLLIY [RASNLES] GIPDRFSGSGSGTDFTLTISSV
      QQKPGQPPKLLIY [RASNLES] GIPARFSGSGSRTDFTLTINPV
      QQKPGQPPKLLIY [WASTRES] GVPDRFSGSGSGTDFTLTISSL

        √                       √     √
      EAEDVATYYC [QQSNEDPLT] FGQGTKLEIKR...
      EADDVATYYC [QQSNEDPLT] FGAGTKLELKR...
      QAEDVAVYYC [QQYYSTPYS] FGQGTKLEIKR...
```

Fig. 13


Competitive Binding of 125I—mIB4 on Human PMNs
◇ mIB4 #13 IC50 = 646pM
◆ Veneer IC50 = 432pM
Mean CPM Bound/1X10^5 PMNs(n=2)
Molar Concentration Added
0
1000
2000
3000
4000
10^-11
10^-10
10^-9
10^-8
10^-7
10^-6

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 92 30 4225

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 88, no. 10, 15 May 1991, WASHINGTON DC, US pages 4181 - 4185 S. GORMAN ET AL. 'Reshaping a therapeutic CD4 antibody.' * the whole document *	1-12	C12N15/13 C12P21/08
D,Y	NUCLEIC ACIDS RESEARCH vol. 19, no. 9, 11 May 1991, LONDON, GB pages 2471 - 2476 B. DAUGHERTY ET AL. 'Polymerase chain reaction facilitates the cloning, CDR-grafting, and rapid expression of a murine monoclonal antibody directed against the CD18 component of leukocyte integrins.' * the whole document *	1-12	
D,Y	NATURE. vol. 332, 24 March 1988, LONDON, GB pages 323 - 327 L. RIECHMANN ET AL. 'Reshaping human antibodies for therapy.' * the whole document *	1-12	TECHNICAL FIELDS SEARCHED (Int. Cl.5) C07K
P,X	EP-A-0 438 312 (MERCK & CO., INC.) * claims *	1-12	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	30 SEPTEMBER 1992	NOOIJ F.J.M.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document